

Capsule Polysaccharide Mediates Bacterial Resistance to Antimicrobial Peptides

Miguel A. Campos,^{1,2†} Miguel A. Vargas,^{1†} Verónica Regueiro,^{1,2}
Catalina M. Llompart,^{1,2,3} Sebastián Albertí,^{1,2,3}
and José A. Bengoechea^{1,2,3*}

Unidad de Investigación, Hospital Universitario Son Dureta,¹ Institut Universitari d'Investigacions en
Ciències de la Salut,² and Àrea de Microbiologia, Departamento de Biología, Universidad de las
Islas Baleares,³ Palma de Mallorca, Spain

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The innate immune system plays a critical role in the defense of areas exposed to microorganisms. There is an increasing body of evidence indicating that antimicrobial peptides and proteins (APs) are one of the most important weapons of this system and that they make up the protective front for the respiratory tract. On the other hand, it is known that pathogenic organisms have developed countermeasures to resist these agents such as reducing the net negative charge of the bacterial membranes. Here we report the characterization of a novel mechanism of resistance to APs that is dependent on the bacterial capsule polysaccharide (CPS). *Klebsiella pneumoniae* CPS mutant was more sensitive than the wild type to human neutrophil defensin 1, β -defensin 1, lactoferrin, protamine sulfate, and polymyxin B. *K. pneumoniae* lipopolysaccharide O antigen did not play an important role in AP resistance, and CPS was the only factor conferring protection against polymyxin B in strains lacking O antigen. In addition, we found a significant correlation between the amount of CPS expressed by a given strain and the resistance to polymyxin B. We also showed that *K. pneumoniae* CPS mutant bound more polymyxin B than the wild-type strain with a concomitant increase in the self-promoted pathway. Taken together, our results suggest that CPS protects bacteria by limiting the interaction of APs with the surface. Finally, we report that *K. pneumoniae* increased the amount of CPS and upregulated *cps* transcription when grown in the presence of polymyxin B and lactoferrin.

The innate immune system is the first host barrier against infectious agents in areas exposed to microorganisms. One of these areas is the respiratory tract, which should be protected from inhaled pathogens without perturbing its inherent structure and function. Moreover, pathogens should be rapidly killed or otherwise they may easily reach the bloodstream, which is the basis of the high morbidity and mortality associated with respiratory tract infections. It is generally accepted that innate defense mechanisms are critical to keeping pulmonary tissues sterile. Although there are several innate systems involved in lung defense, accumulating evidence shows that antibacterial peptides and proteins (APs) present in the airway surface liquid make up the protective front (12). The most abundant antibacterial agents present in the airways are lysozyme and lactoferrin that are secreted by submucosal glands, surface epithelia, and neutrophils (1, 12, 47). Other peptides found in the airway liquid are α -helical defensins, β -defensins, and cathelicidins (1).

In general, APs have broad-spectrum activity. They are active against gram-negative and gram-positive bacteria, fungi, and some enveloped viruses, and because of this they are being extensively studied for the development of new antimicrobial agents (21, 33). There are four structural classes of antimicrobial peptides: the disulfide-bonded β -sheet peptides, the am-

phipathic α -helical peptides, the extended peptides, and the loop-structured peptides (21, 33). Despite their diverse sizes and structures, nearly all antimicrobial peptides have a net cationic (positive) charge, and the three-dimensional folding results in an amphipathic structure (21, 33). A great body of evidence indicates that the electrostatic interaction between antimicrobial peptides and the negatively charged bacterial membranes is critical for bacterial killing (21, 49). However, it is becoming clear that the hydrophobic nature of these peptides also plays an important role in the interaction with the microbial surface (21, 49). In this context, it is not surprising that bacterial resistance to antimicrobial peptides is linked to structural peculiarities that prevent the initial interaction with the bacterial surface (36). One of the best well-known bacterial strategies is the loss of negative surface charges by, for example, covalently adding aminoarabinose to the lipopolysaccharide (LPS) lipid A moiety (17) or by the incorporation of D-alanine to gram-positive cell wall teichoic acids (37).

Klebsiella pneumoniae is a nosocomial pathogen causing infections that range from mild urinary tract infections to severe pneumonia with a high rate of mortality and morbidity (39). Pulmonary infections are often characterized by a rapid clinical course that leaves very little time for an effective antibiotic treatment. This scenario is getting worse due to the increasing occurrence of *K. pneumoniae* multidrug-resistant strains. It is certain that *K. pneumoniae* will encounter APs, and therefore it is reasonable to postulate that *K. pneumoniae* has developed countermeasures that limit the effectiveness of these agents. We report here that *K. pneumoniae* capsule polysaccharide

* Corresponding author. Mailing address: Unidad de Investigación, Hospital Son Dureta, Andrea Doria 55, 07014 Palma Mallorca, Spain. Phone: 34-971-175334. Fax: 34-971-175228. E-mail: jabengoechea@hsd.es.

† M.A.C. and M.A.V. contributed equally to this study.

(CPS) mediates resistance to antimicrobial peptides and proteins by limiting the interaction of the agents with membrane targets.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. pneumoniae* 52145 is a clinical isolate (serotype O1:K2) described previously (30). The isogenic mutants 52K10, nonexpressing CPS, and 52O21, nonexpressing LPS O antigen, have been recently described (9). Bacteria were grown in Luria-Bertani (LB) medium at 37°C. When appropriate, antibiotics were added to the growth medium at the following concentrations: ampicillin at 100 µg/ml; chloramphenicol at 25 µg/ml, and kanamycin at 20 µg/ml. No significant growth differences between strains were found even under iron restriction conditions (data not shown).

Serum resistance. Human sera containing no antibodies to *K. pneumoniae* were obtained from healthy donors, pooled, divided into small aliquots, and stored at -70°C until use. The sera did not react with *K. pneumoniae* cells in dot blotting experiments at dilutions 1/25 through 1/100. Before use, the aliquots were thawed on ice and were never used refrozen. Complement was inactivated by incubating the sera at 56°C for 30 min. For the serum bactericidal assay, bacteria were grown in 5 ml of LB medium in a 15-ml Falcon tube, collected in the exponential phase of growth, and resuspended in phosphate-buffered saline (PBS) to a final concentration of $\sim 10^8$ CFU/ml. Then, 10-µl samples were mixed with 150 µl of PBS and 50 µl of normal serum or inactivated serum to give a final concentration of 24% in serum. The mixtures were incubated at 37°C for 30 min, after which 50 µl of brain heart infusion broth was added to each mixture to stop complement function. The tubes were kept on ice until the contents of each tube were plated on LB agar plates to determine the viable bacterial numbers. The experiments were carried out in duplicate and repeated at least three times. The serum bactericidal effect was calculated as survival percentage, using the bacterial counts obtained with bacteria incubated in inactivated serum as 100%.

Human BALF collection. Bronchoalveolar lavage fluid (BALF) was obtained from healthy subjects by a standard technique (22). Briefly, by using local anesthesia with lidocaine (2% [wt/vol]) to the upper airways and larynx, a fiberoptic bronchoscope was passed through the nasal passages into the tracheas of the subjects. The bronchoscope was wedged in the right middle lobe, and four 60-ml aliquots of prewarmed sterile 0.9% NaCl solution were instilled. This solution was aspirated through the bronchoscope, collected in prechilled glass bottles, and stored on ice. BALF recovery was between 120 and 200 ml. The bottles were centrifuged to eliminate the cells, and a protease cocktail inhibitor (Sigma) was added to the supernatants, which were stored at -80°C. After lyophilization, BALF was resuspended in sterile water to get a 200-fold concentrated solution with respect to the initial bronchoalveolar lavage volume. BALFs obtained from three different subjects were pooled before doing the antimicrobial assay described below. The present study had the approval of the local ethics committee, and written informed consent was obtained.

Antimicrobial peptides and protein sensitivity assay. *K. pneumoniae* strains were grown at 37°C in 5 ml of LB medium and were harvested ($5,000 \times g$, 15 min, 5°C) in the exponential phase of growth, and a suspension containing ca. 2.1×10^5 CFU/ml was prepared in 1% (wt/vol) tryptone-PBS (pH 7.4). Then, 10 µl of this cell suspension was mixed in Eppendorf tubes with various concentrations of antibacterial agents in a volume of 200 µl, followed by incubation at 37°C for 30 min when the effect of polymyxin B and protamine was tested or for 3 h when the effect of lactoferrin was tested (all of them purchased from Sigma). After that, 100 µl of the suspensions was directly plated on LB agar plates. To test the effect of human neutrophil defensin 1 (HNP-1; Sigma) and β -defensin 1 (HBD1; Peprotech), a broth microdilution method was used. Briefly, a bacterial suspension containing 10^5 CFU/ml was prepared in 10 mM PBS (pH 6.5)–0.1% tryptic soy broth (TSB)–100 mM NaCl. Next, 5 µl of this suspension was mixed in Eppendorf tubes with various concentrations of defensins to get a final volume of 25 µl. After 1 h of incubation, the contents of the Eppendorf tubes were plated on LB agar plates.

Colony counts were determined, and results were expressed as percentages of the colony counts of bacteria not exposed to antibacterial agents. All experiments were done with duplicate samples in four independent occasions.

Assessment of outer membrane (OM) damage. Bacteria were grown in 5 ml of LB medium in a 15-ml Falcon tube on an orbital shaker (180 rpm). Cells were harvested ($5,000 \times g$, 15 min, 5°C) in the exponential phase of growth and resuspended in 5 mM HEPES (pH 7.2).

(i) **OM permeability to lysozyme.** A 1-ml portion of bacterial suspension (optical density at 540 nm [OD_{540}] of 0.3) was incubated with different amounts of polymyxin B for 10 min, and then lysozyme (5 µg/ml) was added. After 10 min

of incubation, cell lysis was monitored by determining the decrease in the OD_{500} . Neither lysozyme nor polymyxin B alone produces any OD decrease under these experimental conditions. The results were expressed as percentages of the ODs of controls incubated in the absence of lysozyme and polymyxin B. All experiments were run with triplicate samples from three independently grown cultures of cells.

(ii) **OM permeability to SDS.** A 1-ml portion of bacterial suspension (OD_{540} of 0.3) was incubated with different amounts of polymyxin B and, after 10 min of incubation, bacteria were pelleted ($12,000 \times g$, 5 min) and resuspended in 1 ml of 0.1% sodium dodecyl sulfate (SDS) in 5 mM HEPES (pH 7.2). After 10 min of incubation cell lysis was monitored by the decrease in the OD_{500} . The results were expressed as percentages of the ODs of controls incubated in the absence of polymyxin B. Under these experimental conditions, polymyxin B did not produce any OD decrease. All experiments were run with duplicate samples from three independently grown cultures of cells.

(iii) **OM permeability to NPN.** A bacterial suspension (OD_{600} of 0.5) was prepared in 5 mM HEPES (pH 7.5)–5 µM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). 1-*N*-phenyl-naphthylamine (NPN) was added to 1 ml of this suspension (final concentration, 10 µM), and after 5 min of incubation polymyxin B was added (at concentrations ranging from 16 to 1 U/ml). After 1 min of incubation, 150 µl was transferred to a well of a 96-well, round-bottom enzyme-linked immunosorbent assay plate, and fluorescence was measured by using a microplate fluorescence reader (FLx800; Bio-Tek) with the following settings: an excitation wavelength of 360 nm with a slit width of 40 nm, an emission wavelength 460 nm with a slit width of 40 nm, and a sensitivity of 70. The results were expressed as the fluorescence increase of cells incubated only with NPN, which was arbitrarily set as 1.

Assessment of polymyxin B binding. (i) **Binding of polymyxin B by OM and LPS.** First, 100-µl suspensions containing different amounts of purified OM or LPS, both obtained as previously described (9, 54), were prepared in 2 mM HEPES (pH 7.2) and mixed with 12 µl of a 62.5-µg/ml polymyxin B stock. After 30 min of incubation at 37°C, the suspensions were centrifuged ($12,000 \times g$, 10 min), the supernatant centrifuged two more times under the same conditions, and the unbound polymyxin B was measured in a bioassay (see radial diffusion assay).

(ii) **Binding of polymyxin B by viable cells.** The assay described by Freer et al. (11) was done with minor modifications. Briefly, cells grown as described above were resuspended in 2 mM HEPES (pH 7.2) at ca. 1.25×10^{10} CFU/ml. Then, 100-µl aliquots containing different amounts of cells were mixed with 12 µl of a 62.5-µg/ml polymyxin B stock. After 5 min of incubation at 26°C, the cells with the bound polymyxin B were sedimented ($12,000 \times g$, 10 min), the supernatant was centrifuged two more times under the same conditions, and the unbound polymyxin B was measured in a bioassay (see radial diffusion assay below).

(iii) **Radial diffusion assay.** To detect the unbound polymyxin B, we used a previously described radial diffusion method (26) with minor modifications. Briefly, the indicator bacteria *Escherichia coli* C600 was grown in LB medium and collected in the exponential phase of growth. An underlay gel that contained 1% (wt/vol) agarose, 2 mM HEPES (pH 7.2), and 0.3 mg of TSB powder per ml was equilibrated at 50°C and inoculated with the indicator bacteria to a final concentration of 6.1×10^5 CFU per ml of molten gel. This gel was poured into standard petri dishes and, after polymerization, small 10-µl wells were carved. Aliquots of 5 µl of the supernatants previously obtained were added and allowed to diffuse for 3 h at 37°C. After that, a 10-ml overlay gel composed of 1% agarose and 6% TSB powder in water was poured on top of the previous one, and the plates were incubated overnight at 37°C. The next day, the diameters of the inhibition halos were measured to the nearest 0.1 mm, and after subtracting the diameter of the well, were expressed in inhibition units (10 U = 1 mm).

Quantification of CPS. Cell-associated CPS was purified by a phenol-extraction method (52). Briefly, bacteria were grown in 5 ml of LB medium in 15-ml Falcon tube on an orbital shaker (180 rpm) and harvested by centrifugation ($5,000 \times g$, 15 min, 5°C). The cell pellet was washed once with 1-ml of distilled water and resuspended in 500 µl of distilled water. Viable counts were determined from this suspension by dilution plating. After 2 min of incubation at 68°C, 500 µl of phenol was added, and the incubation was continued for another 30 min. After the mixture cooled, 500 µl of chloroform was added, and the aqueous phase was recovered by centrifugation. The CPS was ethanol precipitated at -20°C and resuspended in 1 ml of distilled water.

CPS was quantified by determining the concentration of uronic acid in the samples by using a modified carbazole assay (7) exactly as described by Rahn and Whitfield (40). All experiments were run with three independently grown batches of bacteria.

Construction of a luciferase reporter strain. The firefly luciferase reporter and Pir-dependent suicide vector were constructed as follows. A DNA fragment

containing the promoter region of the *cps* cluster was amplified by PCR with chromosomal DNA from strain 52145 as a template and *Taq* polymerase (Promega) with the primers PCPS52145f (5'-ATTGTTATTGCATATTTGCTGG-3') and PCPS52145r (5'-GGGGTACCCCATCCATTTTCAGCCCCGCTG-3') (the KpnI site is underlined). The primers were designed by using the published sequence of the *K. pneumoniae* Chedid K2 *cps* cluster (accession no. D21242). The PCR fragment was cloned into pGEM-T Easy (Promega) to obtain pGEMPCps. The cloned fragment was sequenced to ensure that no mistakes were introduced during amplification. The firefly luciferase gene (*lucFF*) coding region was amplified by PCR with pRV34 (6) as a template with the phosphorylated primers BCCP-1 (5'-GAGGAGAAATTAAGTATGAGGGG-3') and BCCP-2 (5'-TTACAATTTGGACTTTCCGCC-3'). Plasmid pGEMPCps was digested with KpnI, blunt ended by using T4 DNA polymerase, and ligated with *lucFF* PCR fragment. A plasmid in which *lucFF* was cloned under the control of the *cps* promoter was identified by restriction digestion analysis and named pGEMPCpsluc. This plasmid was digested with PvuII, and the fragment containing the *cps* promoter region and the *lucFF* was gel purified and cloned into the EcoRV site of the suicide vector pEP185.2 (24) to create pEPPCpsluc. This plasmid was transformed into *E. coli* S17-1 λ pir, which mobilized it into *K. pneumoniae* 52145. A chloramphenicol-resistant transconjugant was selected in which the suicide vector was integrated by homologous recombination, and this was confirmed by Southern blotting (data not shown). Insertion of the suicide vector into the *cps* cluster did not alter CPS production, and the amount of CPS produced by the reporter strain, quantified by determining the concentration of uronic acid, was similar to the one of the wild-type strain.

Luciferase assay. The reporter strain was grown until mid-log phase, pelleted, and resuspended to an OD₆₀₀ of 0.6 in PBS. A 1-ml aliquot was centrifuged (12,000 \times g, 3 min), and the bacterial pellet was resuspended in 500 μ l of bacterial lysis buffer (100 mM potassium phosphate [pH 7.8], 2 mM EDTA, 1% Triton X-100, 5 mg of bovine serum albumin/ml, 1 mM dithiothreitol, 5 mg of lysozyme/ml). After 15 min of incubation at room temperature, cell debris was removed by centrifugation (12,000 \times g, 1 min), and 300 μ l of the supernatant was transferred to a new tube. The bacterial extract was assayed within 20 min. To this end, a 50- μ l aliquot of the extract was mixed with 100 μ l of luciferase assay reagent (Promega). Luminescence was immediately measured with a Triathler luminometer (Hydrex) and expressed as relative luminescence units. To test the effect of antibacterial agents in the expression of the luciferase fusion, the agents were added when the culture reached the exponential phase. After 90 min of incubation, the culture was treated as described above. All measurements were carried out in triplicate in at least two separate occasions.

Statistical methods. Comparisons among groups were made by the two-sample *t* test or, when the requirements were not met, by the Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant.

RESULTS

CPS is involved in antimicrobial peptide resistance. Recently, we have shown that the CPS of *K. pneumoniae* is essential to cause pneumonia in the mouse model (9). Mice infected intratracheally with strain 52145, wild-type strain, developed pneumonia and became bacteremic before death. This course of infection is similar to the one of fatal human infections. In contrast, mice infected with 52K10, the CPS-deficient mutant, did not show any signs of pneumonia, and bacteria were completely eliminated from lungs 2 days after infection (9). In that study, it was shown that phagocytosis of 52K10 by lung macrophages was one of the innate mechanisms to clear the infection.

We decided to explore the contribution of other innate systems, such as the complement system and the antibacterial agents present in the BALF (1, 12, 47), to clear the CPS mutant. However, both strains 52145 and 52K10 were resistant to the bactericidal action of complement (100% relative survival). In contrast, 1 h of incubation in the presence of BALF was enough to kill 52K10 (10% \pm 8% survival), whereas the survival of 52145 was not affected (100% \pm 3% survival). Taking into account that APs are among the array of antibacterial compounds present in BALF (1, 12, 47), we hypothesized

that strain 52K10 could be more sensitive to these agents than 52145. Thus, we tested the sensitivity of both strains to HNP-1, HBD1, lactoferrin, protamine, and polymyxin B. The first three have been shown to be present in BALF (1, 12, 47), whereas the other two have been used by us and others as models of antimicrobial peptide action (5, 15, 18, 41). Confirming our hypothesis, 52K10 was significantly (asterisks in Fig. 1, *P* < 0.05) more sensitive than 52145 to all agents tested. The fact that the agents tested are not structurally related (21, 49) indicates that the sensitivity of 52K10 was not specific to the compound used. Our results also suggested that *K. pneumoniae* LPS O antigen does not play an important role in the resistance to these agents because 52K10 expresses O antigen. Therefore, we hypothesized that an O antigen mutant would be as resistant as the wild type to APs. Indeed, 52O21, a mutant lacking the LPS O antigen but expressing the CPS showed similar resistance to HNP-1, HBD1, lactoferrin, and protamine than 52145 (Fig. 1A to D), whereas it showed an intermediate resistance to polymyxin B (Fig. 1E), one of the most potent APs (49). This last result suggests that LPS O antigen aids CPS to provide protection against this agent.

It is well known that different *K. pneumoniae* strains express different amount of CPS and that, furthermore, this is related to virulence (31, 32). The more-virulent strains produce higher amounts of CPS than the less-virulent ones. On the other hand, antimicrobial peptide resistance has been correlated with virulence (2, 5, 14, 15, 18, 36, 50). Therefore, we hypothesized that *K. pneumoniae* strains expressing less CPS could be more sensitive to antimicrobial peptides than strains expressing higher amounts of CPS. Thus, we studied the sensitivity of a panel of clinical strains (*n* = 12) expressing different amounts of CPS to polymyxin B (Fig. 2A). Even though there were differences in the strains' relative survival in response to polymyxin B, all strains showing <50% survival expressed small amounts of CPS. In fact, there was a significant correlation between the amount of CPS and the relative survival (coefficient of correlation = 0.9).

To further confirm all of these findings, we performed dose-response experiments that allowed us to determine the MICs at which 50% of the isolates are inhibited (MIC₅₀s) of polymyxin B for each strain. The mean MIC₅₀ for strains expressing more than 100 fg of CPS per CFU was 8.0 \pm 0.3 U/ml, whereas the mean MIC₅₀ for strains expressing less CPS than 100 fg per CFU was 1.0 \pm 0.1 U/ml for strains expressing O antigen and 0.2 \pm 0.1 U/ml for the O antigen-negative strains. These results suggest that the mere presence of CPS is not enough to confer resistance to antimicrobial peptides, and it is required that the strains express more than a certain threshold of CPS. Interestingly, of the strains showing low polymyxin B resistance, those expressing LPS O antigen presented higher survival rates indicating, as before, that O antigen may provide some protection against polymyxin B. In turn, this suggests that CPS could be the only factor conferring protection against polymyxin B in strains lacking O antigen. Indeed, a CPS-deficient mutant from strain USA0352/78 showed a dramatic decrease in polymyxin B resistance compared to the wild-type strain (Fig. 2B).

CPS affects the antimicrobial peptides' function as OM permeabilizers. An important feature of the action of antimicrobial peptides on gram-negative bacteria is the disorganization of the OM (21, 49). This is not their lethal action but it is their

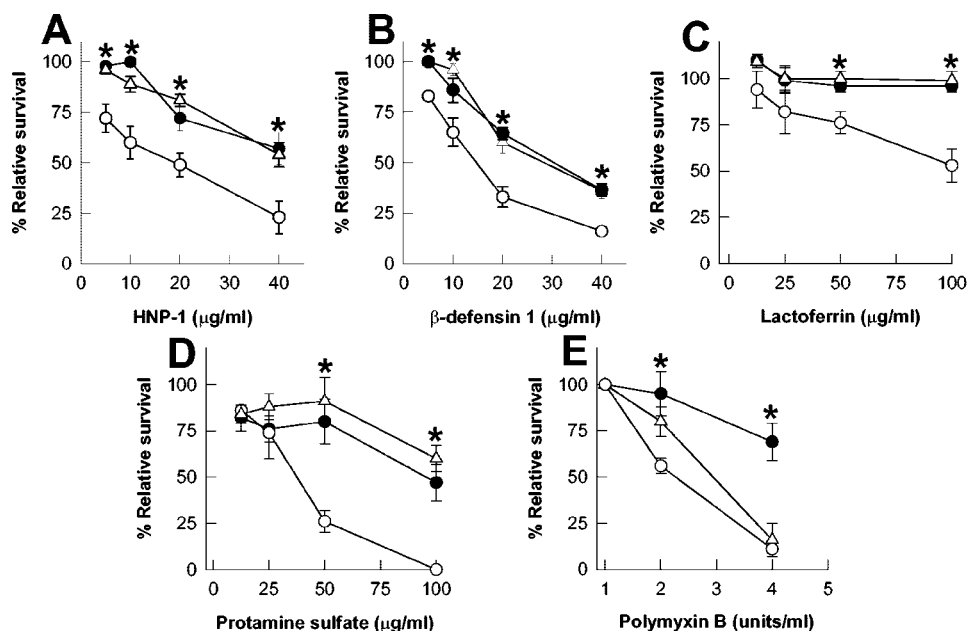


FIG. 1. Role of CPS in the resistance to antimicrobial peptides and proteins. Survival of bacteria (percentage of colony counts of cells not exposed to the agents) with different amounts of HNP-1 (A), HBD1 (B), lactoferrin (C), protamine sulfate (D), and polymyxin B (E). Each point represents the mean and standard deviation of eight samples from four independently grown batches of bacteria, and significant survival differences between 52145 and 52K10 are indicated by asterisks. Symbols: ●, *K. pneumoniae* 52145; △, *K. pneumoniae* 52021; ○, *K. pneumoniae* 52K10.

way of permeating the OM to reach their final target inside the cell. After the peptides bind to the LPS anionic sites the membrane is thought to develop transient “cracks,” which allow the entrance of a variety of molecules normally excluded, including the peptide itself. This is called the self-promoted pathway (21). Our previous findings showing that strain 52K10 was more sensitive to antimicrobial peptides than the wild type led us to explore the possibility that the self-promoted pathway is increased in this strain. To measure OM disturbance, we used as probes the bacteriolytic anionic detergent SDS and lysozyme (a lytic enzyme acting in the periplasm). The OM efficiently excludes both probes, and their entrance can be easily monitored because they cause cell lysis (detected as a drop in turbidity). Polymyxin B was used as a model of antimicrobial peptide because its permeabilizing action is well established (49). It is known that the amount of agent required for the bactericidal effect is less than that necessary to cause membrane damage (10, 49). Figure 3 shows that the SDS- or lysozyme-induced cell lysis was higher in strain 52K10 than in 52145 (Fig. 3A and B, respectively), indicating that the OM-sensitizing action of polymyxin B was more profound on 52K10 than 52145. Viability at the end of the incubation in the presence of lysozyme was $100\% \pm 5\%$ for 52145 and $44\% \pm 9\%$ for 52K10. As a third probe to detect OM disturbance, we used NPN, which is an uncharged hydrophobic fluorescent probe that fluoresces weakly in an aqueous environment but strongly in the hydrophobic interior of a membrane (5, 27, 49). When NPN is added to cells, it fluoresces weakly since it is unable to breach the OM barrier and, in addition, it is a substrate for efflux pumps. However, upon OM destabilization in the presence of an energy inhibitor, NPN partitions into the membrane, emitting a bright fluorescence. The results shown in Fig.

3C demonstrate that polymyxin B significantly (asterisks, $P < 0.05$) increased NPN partition into the OM of strain 52K10 but not into the OM of strain 52145.

CPS reduces the binding of antimicrobial peptides to the bacterial surface. Thus far, we have shown that *K. pneumoniae* CPS mutant is more sensitive to the action of APs than the wild-type strain. However, our results still did not reveal whether CPS plays a direct role. It is possible, for example, that the absence of CPS causes structural changes in the OM, thereby allowing an easier interaction of the agents with the bacterial surface. In this scenario, the role of CPS in the resistance to antibacterial agents would be indirect. To examine this possibility, we measured the amount of polymyxin B adsorbed by OMs or LPSs purified from 52145 and 52K10. No significant differences were found between the amounts of polymyxin B adsorbed by purified OMs and LPSs of both strains (Fig. 4A and B). The OM protein and LPS pattern of both strains studied by SDS-PAGE were similar (data not shown). In addition, matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis of lipid A extracted from both strains revealed no differences between them (unpublished findings). Taken together, the results indicated that 52K10 sensitivity to APs was not due to gross changes in the OM architecture. Then, we reasoned that CPS would play a direct role in the resistance to antibacterial agents by limiting their interaction with the OM. If this is so, the CPS mutant should bind more APs than the wild-type strain. The results shown in Fig. 4C demonstrate that 52K10 bound significantly more polymyxin B than 52145 (asterisks, $P < 0.05$), thereby giving experimental support to our hypothesis. Under these experimental conditions, purified CPS, tested at up to 400 µg/ml, did not bind any polymyxin B.

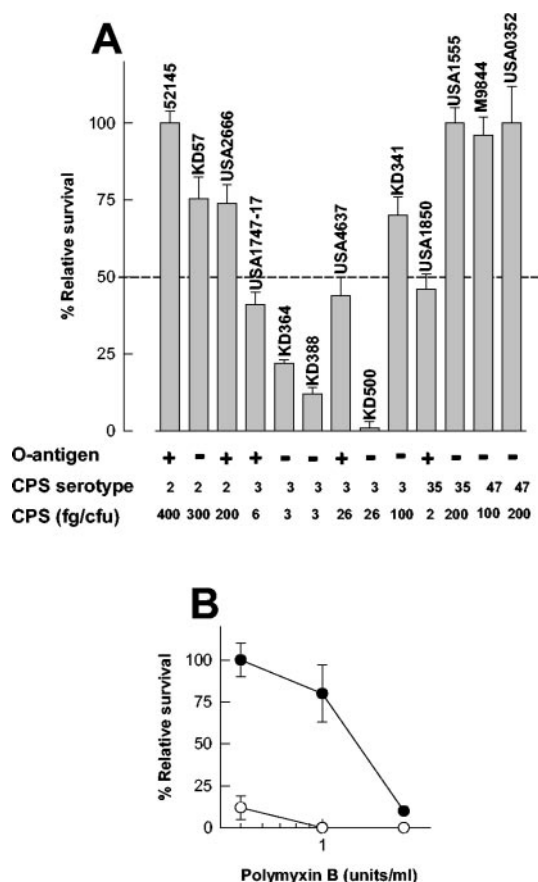


FIG. 2. Role of CPS and O antigen in the resistance to antimicrobial peptides. (A) Survival of bacteria (percentage of the colony counts of cells not exposed to polymyxin B) after incubation with 1 U of polymyxin B/ml. Error bars display the standard deviation from the mean of three experiments, each one run in duplicate. Also shown are the CPS serotype, the amount of capsule bound to the cell surface, and whether the strains tested express O antigen. (B) Survival of bacteria (percentage of colony counts of cells not exposed to polymyxin B) after incubation with different amounts of polymyxin B. Each point represents the mean and standard deviation of four samples from two independently grown batches of bacteria. Symbols: ●, *K. pneumoniae* USA0352/78 (wild-type strain); ○, *K. pneumoniae* USA0352/78-3 (CPS mutant).

Antimicrobial peptides and proteins modulate CPS expression. It is likely that *K. pneumoniae* will encounter APs in vivo, and therefore we sought to determine whether APs may contribute to environmental signals that trigger changes in CPS expression. To address this issue, we quantified the amount of capsule expressed by 52145 after 3 h of incubation in the presence of APs by measuring uronic acid, a component of the K2 CPS (Table 1). 52145 incubated in the presence of protamine sulfate expressed the same amount of CPS as bacteria incubated without the agent, whereas bacteria incubated in the presence of polymyxin B or lactoferrin expressed more CPS than bacteria incubated without the agents. To test whether APs increase *cps* transcription, we introduced the *cps::lucFF* transcriptional fusion into 52145 and then monitored light production in the presence or absence of APs (Table 1). Incubation in the presence of protamine sulfate did not alter *cps* transcription, whereas polymyxin B and lactoferrin significantly

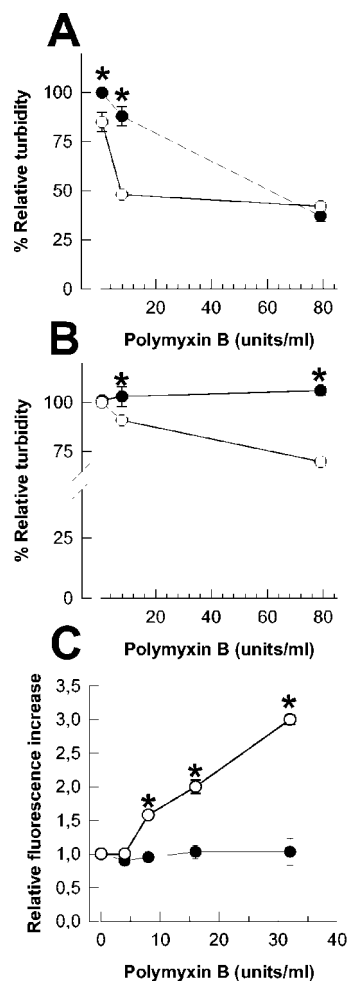


FIG. 3. CPS affects the self-promoted pathway. Effect of polymyxin B on the permeability of the OM determined as SDS-induced lysis (A), lysozyme-induced lysis (B), and NPN partition (C). In the absence of polymyxin B, *K. pneumoniae* 52145 took up more NPN ($17,560 \pm 200$ RLU) than *K. pneumoniae* 52K10 ($10,450 \pm 200$ RLU). Each point represents the mean and standard deviation of four samples from two independently grown batches of bacteria, and significant differences between 52145 and 52K10 are indicated by asterisks. Symbols: ●, *K. pneumoniae* 52145; ○, *K. pneumoniae* 52K10.

(asterisks, $P < 0.05$) increased the expression of the transcriptional fusion.

DISCUSSION

The main novel finding described here is that bacterial CPS is involved in the resistance to APs. We demonstrated that *K. pneumoniae* CPS mutant is more sensitive to APs than the wild-type strain and binds more polymyxin B than the wild type with a concomitant increase in the self-promoted pathway. Taken together, the results strongly suggest that CPS limits the interaction of APs with the bacterial surface. As far as we are aware, it is the first time that this mechanism of resistance to APs has been reported. It is important to point out that the CPS mutant was sensitive to three agents constitutively found in human airway fluid, HNP-1, HBD1, and lactoferrin (1, 12, 47). Furthermore, the concentrations tested are within the

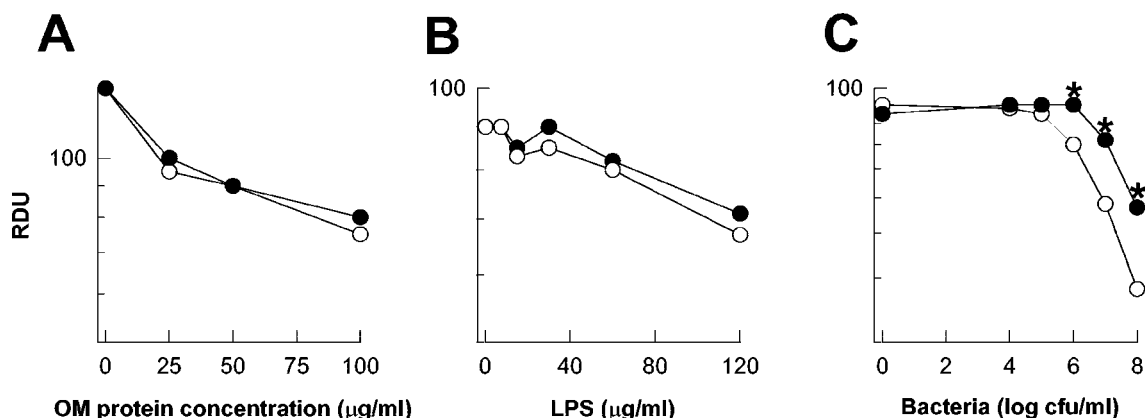


FIG. 4. CPS affects the binding of polymyxin B to the cell surface. Binding of polymyxin B to purified OM (A), purified LPSs (B), and bacteria (C) is shown. Each point represents the mean and standard deviation (covered by the symbol) of four samples, and significant differences between 52145 and 52K10 are indicated by asterisks. Symbols: ●, *K. pneumoniae* 52145; ○, *K. pneumoniae* 52K10.

range reported to be present in the respiratory fluid of healthy subjects (1, 12, 47), thereby giving physiological relevance to our work.

Gram-negative bacteria have developed a variety of strategies to withstand APs. *E. coli* and *Salmonella enterica* serovar Typhimurium possess an OM protein, OmpT, that proteolytically cleaves APs (16, 45). *Neisseria gonorrhoeae* and *Yersinia enterocolitica* are endowed with energy-dependent efflux systems that pump APs out of the cell (6, 42). The *sap* genes, encoding proteins similar to those involved in ATP-binding cassette (ABC) transport systems and potassium transporters, have been shown to play a role in AP resistance (34, 35). However, it is generally considered that the most important mechanisms of resistance to APs involve surface modifications and specifically those in the LPS molecule (14, 36). In serovar Typhimurium these modifications include (i) the addition of aminoarabinose to the phosphate groups at the lipid A level; (ii) replacement of the myristate acyl group of the lipid A by 2-OH-myristate; and (iii) the formation of hepta-acylated lipid A by the addition of palmitate (17–20). Expression of these changes is transcriptionally regulated through the two-component signaling systems *phoP/phoQ* and *pmrA/pmrB*, which sense environmental signals such as low pH, magnesium concentration, or iron availability (13, 43, 53). However, the current evidence also indicates that resistance to APs is impaired

if the LPS lacks the O antigen even though the described lipid A modifications are present. It is believed that the LPS O antigen acts as a shield by hindering the accession of APs to inner LPS targets (2, 8, 28, 38, 44, 48, 50). This is reminiscent of the role that O antigen plays in serum resistance (46). The findings reported in the present study experimentally support that *K. pneumoniae* CPS can also act as a shield against APs. In fact in strains lacking the LPS O antigen, CPS seems to be the only shield hindering the accession of APs. It is important to note that our findings were not related to the CPS serotype or chemical composition (see Fig. 2A) but are dependent on the amount of CPS expressed by the strains. If this is so in other capsulated bacteria, as we believe, this might be the reason why other authors did not find in their studies that CPS protects against APs (25, 51). In addition, it should be considered that CPS expression could be regulated, thereby affecting the degree of protection conferred against APs. For example, in our experience it is important to assay bacteria taken in the exponential phase of growth because at least *K. pneumoniae* and *E. coli* significantly downregulate CPS production in the stationary phase of growth (M. A. Campos and J. A. Bengoechea, unpublished results).

It is known that *K. pneumoniae* strains expressing less CPS are less virulent than strains expressing higher amounts of CPS (31, 32) and, furthermore, that CPS mutants are avirulent in the mouse model of pneumonia (9). To explain this, it is generally accepted that innate defense mechanisms are the main players to clear the infection. The results shown in Fig. 2A suggest that APs present in BALF could be one of these innate mechanisms. In fact, when it is taken into account that *K. pneumoniae* strains expressing low amounts of CPS are resistant to complement-mediated killing and phagocytosis by macrophages and neutrophils (3), APs could be considered the front line of defense and perhaps the only innate weapon to kill these strains. In this context, we believe that our findings further highlight the importance of APs for eliminating lung pathogens. Recent studies have provided direct evidence supporting this idea. Mouse knockouts defective in one AP present in BALF are more susceptible to infections caused by gram-negative and gram-positive bacteria (4, 29).

TABLE 1. Effect of antimicrobial peptides on capsule polysaccharide production and transcription^a

| Induction condition | Mean \pm SD | |
|---|--|-----------------|
| | Amt of capsule ($\mu\text{g}/10^4$ CFU) | RLU |
| None | 76.3 \pm 7 | 1,269 \pm 80 |
| Protamine sulfate (50 $\mu\text{g}/\text{ml}$) | 78.0 \pm 5 | 1,223 \pm 50 |
| Polymyxin B (0.5 U/ml) | 114.1 \pm 4* | 1,708 \pm 40* |
| Lactoferrin (100 $\mu\text{g}/\text{ml}$) | 108.4 \pm 10* | 2,989 \pm 97* |

^a The amount of capsule was quantified by measuring uronic acid (a component of the K2 polymer). Luciferase activity was measured in a strain carrying the transcriptional fusion *cps::lucFF* and expressed as relative luminescence units (RLU). *, Significantly different from the result in the absence of inducing conditions ($P < 0.05$, [two-tailed *t* test]).

One of the most striking findings in our study is that the presence of polymyxin B and lactoferrin increased the amount of CPS bound to the cell surface and upregulated the *cps::lucFF* transcriptional fusion. This may happen in vivo because lactoferrin is found in BALF and the amount tested is the one reported to be present in BALF of healthy subjects (1, 12, 47). Experiments are under way to confirm this issue. Interestingly, Held et al. (23) reported that two antibiotics, ceftazidime and ciprofloxacin, stimulate CPS production. In light of our findings, it is tempting to postulate that antibiotic treatment may render *K. pneumoniae* more resistant to APs due to the increase expression of CPS. Future studies will address this possibility. On the other hand, a tantalizing hypothesis could be that *K. pneumoniae* upregulates CPS expression in response to harmful agents such as antibiotics and APs. At present, we can only speculate on the molecular mechanisms behind this regulation. This is even more difficult because the regulation of CPS is still poorly understood. We are currently studying how *K. pneumoniae* senses the presence of antimicrobial peptides and how the signal is transduced in order to increase the production of CPS.

Finally, it is worthwhile commenting on the biological implications of our findings. CPS is one of most important virulence factors of bacteria, both gram-negative and gram-positive, and also fungi. The current evidence obtained with different pathogens indicates that CPS is involved in resistance to complement-mediated killing and neutrophil-mediated phagocytosis. Of note, APs are also present in the tissues, such as the gut or the skin, colonized by these pathogens. We put forward the hypothesis that CPS involvement in AP resistance could be a general feature of capsulated bacteria provided that the amount of CPS expressed is above a certain threshold, as we have shown in the case of *K. pneumoniae* (Fig. 2A). In support of this hypothesis we have recently found that a *Streptococcus pyogenes* CPS mutant, strain TX72, is more sensitive to antimicrobial peptides than the heavily capsulated wild-type strain (J. A. Bengoechea, unpublished results).

On the other hand, we believe that our findings may also open new avenues of research for designing new antibacterial agents. The use of compounds that inhibit CPS production might be useful to render capsulated bacteria sensitive to innate defense mechanisms. One of our goals in the near future is the identification of CPS biosynthesis inhibitors.

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